

Investigating genes encoding membrane
proteins in grapevine (*Vitis vinifera* L.) and
Vitis spp. rootstocks to determine their role in
chloride exclusion

By
Sam William Henderson
B. Biotech. (Hons.)

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Faculty of Sciences
School of Agriculture, Food and Wine
The University of Adelaide

October 2014

Table of contents

Abstract	iii
Declaration	v
Acknowledgements	vi
List of Abbreviations	vii
Chapter 1 Literature review	1
1.1 Background.....	1
1.2 Effect of salt on plants	2
1.3 Effect of salt stress on grapevine	3
1.3.1 Ionic causes and effect on grapevine physiology	3
1.3.2 Effect of salt on grapevine yield	4
1.3.3 Effect of salt on wine production.....	4
1.4 Management of salinity for viticulture	5
1.5 Chloride excluding grapevine rootstocks	5
1.5.1 Differences in chloride accumulation between rootstocks	5
1.5.2 Inheritance of the chloride exclusion trait	6
1.5.3 Mechanism of chloride exclusion	7
1.6 Chloride homeostasis in plants	8
1.6.1 Active versus passive transport.....	8
1.6.2 Regulation of xylem loading of chloride	9
1.7 Crucial genes for plant chloride homeostasis	10
1.8 Identifying genes for chloride exclusion in other crops	12
1.9 Tools for characterisation of grapevine genes	13
1.10 Conclusions.....	14
1.10.1 Experimental aims	15
Chapter 2 Shoot chloride exclusion and salt tolerance in grapevine is associated with differential ion transporter expression in roots	18
Abstract.....	19
Introduction.....	20
Materials and methods	23
Results.....	28
Discussion.....	34
References.....	38
Figure Legends	47
Supporting information.....	52

Chapter 3	A <i>trans</i>-Golgi localised cation-chloride cotransporter from grapevine (<i>VvCCC</i>) complements an <i>Arabidopsis ccc</i> mutant but does not have a role specific to salt tolerance.	60
Abstract		61
Introduction		62
Materials and methods		65
Results		70
Discussion		75
References		78
Figure legends		82
Supporting information		94
Chapter 4	Overexpression of two grapevine chloride channels restores impaired stomatal movements in a salt-insensitive <i>Arabidopsis clcc</i> mutant.	108
Abstract		109
Introduction		110
Results		113
Discussion		118
Methods		122
References		126
Figures		130
Supplementary files		133
Chapter 5	Conclusions and future research directions	147
5.1	Background	147
5.2	Gene identification by microarray hybridisation	147
5.3	Gene characterisation	149
5.3.1	<i>VvCCC</i>	149
5.3.2	<i>VvCLC</i>	151
5.3.3	<i>Arabidopsis thaliana</i> as a model for chloride exclusion	152
Appendix I	– Additional analyses of transcriptional events in three grapevine genotypes under salt-stress	153
Appendix II	– Electrophysiological characterisation of <i>VvCLCg</i>	165
Bibliography		169

Abstract

Plant growth and productivity is limited when sufficient salt (NaCl) accumulates in the soil solution to impose an osmotic stress, and when the concentrations of the dissociated sodium (Na^+) and chloride (Cl^-) ions accumulate to toxic levels in cells and tissues. One mechanism associated with plant salt tolerance is the ability to limit Na^+ and Cl^- accumulation in the shoot. The aim of this thesis was to investigate the genetic basis of shoot Cl^- exclusion by using the Cl^- sensitive dicotyledonous crop grapevine (*Vitis vinifera* L.).

Root transcriptomes of three *Vitis* genotypes were compared by microarray hybridisation. Genotypes were selected for their contrasting abilities to exclude Cl^- from shoots: 140 Ruggeri (Cl^- excluder), Cabernet Sauvignon (Cl^- intermediate) and K51-40 (Cl^- includer). The magnitude of transcriptional changes due to a 4 day, 50 mM Cl^- treatment were proportional to the amount of the Cl^- accumulating in leaves, with those in Cabernet Sauvignon and K51-40 indicative of secondary stresses, such as the generation of reactive oxygen species. In the Cl^- excluder 140 Ruggeri transcriptional changes were typical of plant primary responses to salt stress, such as the up-regulation of heat shock associated proteins and transcription factors. Transcripts encoding anion channels, that may potentially regulate Cl^- transport from root to shoot in grapevine, were not significantly differentially expressed in any genotype due to salt-stress. This was consistent with the observation that Cl^- exclusion in grapevine is not a salt-inducible trait. In contrast, a comparison of transcriptional differences between K51-40 and 140 Ruggeri in the absence of salt-stress revealed a list of gene candidates. Many of these genes were homologous to anion channels and their regulators from guard cells that facilitate stomatal closure in *Arabidopsis thaliana*. Genes included *VvSLAH3*, *VvALMT1*, *VvSnRK2.6* and members of the *NRT1* family.

There are a number of genes, not regulated at the transcriptional level by salt, which have been implicated in salt tolerance in previous studies of *Arabidopsis thaliana*. The role of some homologous genes in grapevine was also investigated in this thesis with the aim of clarifying their putative roles in shoot Cl^- exclusion. To this end, the functional properties of a cation-chloride cotransporter (*VvCCC*) and two chloride channels (*VvCLCc1*, *VvCLCg*) were examined. *VvCCC* functionally complemented an *A. thaliana ccc* deletion mutant reversing a stunted-growth phenotype and perturbed Cl^- homeostasis. However, membrane localisation data indicates that *VvCCC* is likely to be localised to the *trans*-Golgi network so may not directly mediate xylem loading or retrieval of Cl^- . Investigation into plant CLCs revealed that

an *Arabidopsis clcc* mutant was not salt-sensitive, which contrasts with previous reports. When overexpressed, neither *VvCLCc1* nor *VvCLCg* could improve growth of *Arabidopsis* or yeast on salt containing media. However both *VvCLC* isoforms could restore impaired stomatal functioning in the *Arabidopsis clcc* mutant. Together these data suggest that plant CCCs and CLCs are not crucial genes for Cl^- exclusion and salt tolerance despite previous speculation. Future investigation into plant Cl^- exclusion will benefit from the candidate genes identified in the transcriptional screen of this thesis.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Sam Henderson and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

.....
Sam William Henderson

.....
Date

Acknowledgements

I thank my supervisors Dr Matthew Gilliham, Professor Steve Tyerman and Dr Rob Walker for guidance during the course of this study, for critically reading my work, and for supporting my research ideas. My principal supervisor, Matthew Gilliham, is especially thanked for encouraging me to commence this project, and for continued support through the many hours spent discussing results and further experiments.

I also thank the Grape and Wine Research and Development Corporation for generously providing financial support to this project, and for supporting my travel to present this work at international conferences. I thank the Australian government for providing financial support through an Australian Postgraduate Award, and the University of Adelaide School of Agriculture, Food and Wine for providing resources necessary to carry out this project and opportunities for casual teaching.

This work would not have been possible without input from various people. I thank Deidre Blackmore for cultivating the grapevine in hydroponics, for setting up the grapevine rooted leaf experiments and organising the plant harvests. Mandy Walker is acknowledged for providing valuable advice and for critically reading my work. I am immensely grateful to Ute Baumann for processing the raw microarray files and performing many different analyses. Our collaboration was invaluable to me, taught me much about bioinformatics, and made me realise how much is left to learn.

I thank Sunita Ramesh for providing guidance and sharing knowledge of all things related to *Xenopus* oocytes. I also thank my colleagues in the Plant Research Centre Brad Hocking, Jiaen Qui, Asmini Athman, David Chaisson, Bo Xu, Wendy Sullivan, Johannes Scharwies and Brent Kaiser for help and advice at various times throughout this project.

Finally I thank my wife Virginie for her patience and for providing me with an enormous amount of support and encouragement during my pursuit of this degree. I will always appreciate it.

List of Abbreviations

140R	140 Ruggeri
ABA	abscisic acid
BLAST	Basic Local Alignment Search Tool
°C	degrees Celsius
CaMV35S	cauliflower mosaic virus constitutive promoter
cDNA	complementary DNA
CFP	cyan fluorescent protein
Cl ⁻	chloride
Col-0	<i>Arabidopsis thaliana</i> ecotype Columbia-0
cRNA	capped RNA
CS	Cabernet Sauvignon
DNA	deoxyribonucleic Acid
dNTP	deoxyribonucleotide triphosphate
kg, g, µg, ng, pg	kilograms, grams, micrograms, nanograms, picograms
hr	hour
K ⁺	potassium
kb	kilobase
L, mL, µL, nL	litre, millilitre, microlitre, nanolitre
Log ₂ FC	fold change transformed to the base 2 logarithm
mg, µg, ng	milligrams, micrograms nanograms
M, mM, µM, nM	molar, millimolar, micromolar, nanomolar
mRNA	messenger RNA
mV	millivolts
Na ⁺	sodium
NO ₃ ⁻	nitrate
PCR	polymerase chain reaction
PEG	polyethylene glycol
qRT-PCR	quantitative real time PCR
Rb ⁺	rubidium
RNA	ribonucleic acid
RT	reverse transcriptase
Spp.	species
T-DNA	transfer DNA

TGN	<i>trans</i> -Golgi network
TFP	teal fluorescent protein
V.	<i>Vitis</i>
v/v	volume per volume
w/v	weight per volume
Ψ	water potential
YFP	yellow fluorescent protein
Zn ²⁺	zinc